TO EVALUATE THE PROTECTIVE EFFECT OF PROTEIN OF PIPER LONGUM LINN ON CELL VIABILITY OF HUMAN PERIPHERAL LYMPHOCYTES AGAINST DAMAGE INDUCED BY HYDROGEN PEROXIDE AND TERTIARY BUTYL HYDROPEROXIDE

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ABSTRACT:

A major alkaloid, piperine is present in P.longum which showed significant anti-metastasis activity. Chemopreventive effects are also shown by piperine when it is administrated orally on the animals suffering from lung cancer. The fruits were air dried pulverized and used for analysis. Preliminary screening of secondary metabolites. Natural plants like long pepper are the main sources of bioactive molecules and have played a major role in discovery of lead compounds .The curative properties of medicinal plants of long pepper are due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, sterols etc. The bio enhancing effect of pepper species in traditional as well as modern medicine may be due to two major mechanisms; first, because of a nonspecific mechanism in which the rapid absorption of the drug may be observed by the decreased secretion of HCl. Despite the outstanding potential of piperine reported in pre-clinical studies, no clinical trials are ongoing in cancer patients. The bio enhancing properties of piperine are being explored in a clinical trial in association with curcumin to assess whether the combination may reduce inflammation and discomfort from a ureteric stent in cancer patients

Key Words: *P. longum, Peripheral blood samples, hydrogen peroxide.*

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INTRODUCTION

Long pepper or Pippali *(Piper longum* Linn.L; family Piperaceae) is a very important medicinal plant. It is indigenous to South-India and Western Ghats of India, and is cultivated in hotter parts of India mainly Orissa, Kerala and Central to North Eastern Himalayas. It is being used from thousands of years in various traditional medicinal practices such as Ayurveda, Unani etc. *Piper longum* also shows various pharmacological activities such as antifungal, insecticidal, antimicrobial, antiamoebic, antidiabetic, antioxidant, anti-cancerous and effect on respiratory system. Current review deals with the Botany, phytochemistry and pharmacology of *P. longum*. In traditional medicinal practices i.e. Chinese traditional medicine

MATERIALS AND METHODS

The methodology of research shows the general pattern of organizing the procedure for gathering valid and reliable data for the purpose of investigation.

Blood Collection and Lymphocyte Isolation and Culture

Peripheral blood samples obtained from healthy volunteer donors with an average age of 28 that did not smoke, drink, or use chronic medication were collected after 12 h overnight fasting by venipuncture using a top Vacutainer® (BD Diagnostics, Plymouth, UK). The study was approved by the Ethic Committee of the Regional Center of Blood Transfusion of medical council. Peripheral blood lymphocytes were isolated under sterile conditions by using a density gradient present in the Histopaque-1077 reagent, according to the study by Gafrikova et al.. Five milliliters of blood were mixed in Eppendorf tubs with the same volume of phosphate buffer solution. Histopaque-1077 (100 L) was underlayed and tubs were spun at 800g for 30 min at 4 C. Lymphocytes were retrieved from just above the boundary between the phosphate buffer and Histopaque-1077. One hundred microliters of lymphocytes were mixed with 1 mL of PBS in new Eppendorf tubes and spun again at 800g for 5 min at 4 C. Supernatant was removed and the lymphocytes were counted in a Neubauer chamber using Trypan blue (0.4%). Cell viability of about 98% was accepted for further experiments. Lymphocytes were cultured in culture medium containing 1 mL RPMI 1640 supplemented with 10% of fetal bovine serum and 1 mM of gentamycin. Cells were maintained in a suspension culture at37 C in a humidified 5% CO2 atmosphere.

Cell Treatment and MTT Assay

To assess the effect of fruit pulp extract on human lymphocyte viability (cytotoxicity), cells were incubated in 96-well plates (5 _ 103 cells /well) for 1 h with vehicle (culture medium RPMI) or with different concentrations of extract (100–1000 _g/mL), and the percentages of viable cells in the extract treatment were compared to the vehicle. Furthermore, to evaluate the potential of the extract to protect human lymphocytes from oxidative damage (cytoprotection), cells were incubated (1 h, 37 _C, 5% CO2) with samples at different concentrations (100–1000 _g/mL) before exposure to the hydrogen peroxide or to tert-butyl hydroperoxide cytotoxic actions.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as described previously for evaluating the cytotoxicity and the cytoprotective effects of the extract. Briefly, fresh medium (100 _L) containing MTT (0.5 mg/mL) was added into each well containing pretreated cells and incubated for 2 h at 37 _C. After removing MTT-containing medium, 100 _L of dimethyl sulfoxide (DMSO) was added into each well to dissolve the purple formazan crystal formed in viable cells. The plates were then shaken gently for 20 min in darkness and absorbance was measured at 595 nm on a microtiter plate reader. Ascorbic acid was used as the standard and the experiment was performed in triplicate.

The Comet assay for determination of DNA damage

Lymphocytes were treated with MYR B (10 µM) and MYR N (20 µM) in combination with H2O2 (50 µM) for 1 h. The cell suspension was centrifuged at 3000 rpm (1000 g). The supernatant was removed and the pelleted cells were subjected for the Comet assay as previously defined with some changes

Measurement of ROS accumulation in isolated lymphocytes

Isolated lymphocytes were grown in a 96-well plate overnight. Cells were washed with phosphate buffer saline (PBS) prior to addition of various chemical treatments in PBS and incubated for 1 h at 37 °C in the presence of 5% CO2. Once incubation was over, the cells were washed again with PBS following the loading of a fluorescent probe, DCFDA dye (Abcam, UK) into each well and incubated again for 45 min under the same conditions. Then dye was replaced by 100 µl of PBS and fluorescence was measured at 485/535 nm using Promega Glomax explorer version 2.4. Data were analysed using the Graph Pad Prism 7.02.

Assay of cellular enzyme and total thiol content

Reduced glutathione (GSH) levels and oxidised glutathione (GSSG) contents in experimental and normal lymphocytes were analysed using the GSH/GSSG Ratio Detection Assay Kit (Fluorometric—Green) (Abcam, UK) according to the manufacturer protocol (25). Briefly, isolated lymphocytes were harvested overnight in 6-well plate then treated with chemicals for 1 h. Cells were washed with cold PBS and re-suspended in 100 µl of cold lysis buffer supplemented with 10 µl of protease inhibitors then thoroughly mixed by pipetting and centrifuged at 400g for 5 min to remove insoluble. The supernatant (sample) was collected and kept on ice for further use.

50 µl of each GSH and GSSG standards (kit components) were added to 96-well plates in duplicates. For GSH detection, 50 µl of the GSH assay mixture (GAM) (provided with the kit) was added to each GSH standard and sample. For GSSG detection, total GSH assay mixture (TGAM) was added to each GSSG standard and sample. Incubated for 60 min at room temperature in the dark and then fluorescence was measured at 490/520 nm using Promega Glumax explorer version 2.4. Data were analysed using Graph Pad Prism 7.02.

RESULTS

Results presented show the antioxidant activity of OLP and HT determined with ABTS·+ method. Their scavenging effect on ABTS·+ radical was concentration-dependent (R2 = 0.982) with lower activity of OLP than HT. Results obtained in our study are in accordance with the previously published reports. The reducing capacity of OLP and HT in our study are shown (R2 = 0.988). We observed differences between antioxidant activities measured by these assays; however, these differences could be attributed to different mechanisms of action of tested phenolic compounds. In general, the antioxidant and radical-scavenging activity of phenolic compounds such as oleuropein and hydroxytyrosol is based on the presence of free hydroxyl groups in their chemical structure, catechol phenolic group being a characteristic pattern in both OLP and HT. Important for antioxidative activity are the number and location of aromatic hydroxyl groups of natural polyphenols, as well as stability of the formed aroxyl radicals. The increased reducing power is likely to be correlated with the antioxidant properties of natural compounds due to their hydrogen-donating ability. Fe3+ reduction is an indicator of electron-donating activity which is an important antioxidant mechanism of action of phenolic compounds. It has been reported that the antioxidant capacity of hydroxytyrosol is due to its H-donor ability and the formation of a highly stable aromatic ring radical when losing a hydrogen atom for conjugation. On the other hand, oleuropein shows antioxidant properties due to the *o*-dihydroxy (catechol) structure present in its moiety.

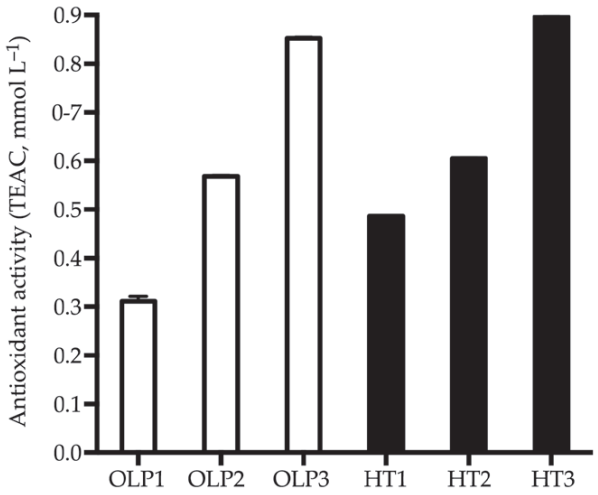

Figure .1. Antioxidant capacities of phenolics oleuropein (OLP) and hydroxytyrosol (HT) determined by ABTS·+ method and expressed as Trolox equivalent antioxidant capacities (TEAC); OLP1, HT1 – 1 µmol L–1, OLP2, HT2 – 2 µmol L–1, OLP3, HT3 – 3 µmol L–1. Mean ± SD, n = 3.

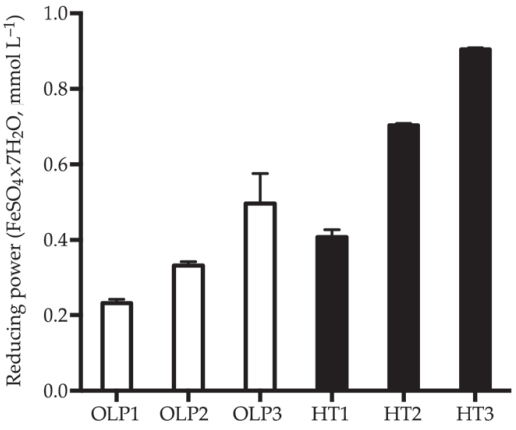

Figure .2 Reducing power of phenolics oleuropein (OLP) and hydroxytyrosol (HT) expressed as FeSO4 × 7 H2O. OLP1, HT1 – 1 µmol L–1, OLP2, HT2 – 2 µmol L–1, OLP3, HT3 – 3 µmol L–1. Mean ± SD, *n* = 3.

Several studies have investigated the ability of OLP and HT to scavenge DPPH radicals and ABTS·+; however, limited data exist against other radicals. Therefore, antioxidant activity was also determined with the CUPRAC method. In comparison with other assays, the advantage of the cupric reducing method is that the measurement of antioxidant capacity is carried out at nearly physiological pH; therefore, it mimics the physiological Action of the antioxidants. Additionally, it is suitable for the determination of hydrophilic and lipophylic antioxidants (24). Potent antioxidant capacity of tested compounds was confirmed with the cupric reducing method; results are presented (*R*2 = 0.996)

DISCUSSION

Further the extract was subjected to analyze for its antioxidant ability against Hydroxyl radical scavenging activity and inhibition of formation of lipid peroxidation. Here, Alpha tocopherol, BHA and Ascorbic acid are used as standard antioxidants at 400µM concentration and 10µg of Long Pepper protein extract was used. When compared to the standards, Long pepper proteins showed an inhibitory activity of 81% and 83% and the results are very promising. Further the extract and Ascorbic acid were subjected to thermal stability test by keeping the extract in boiling water bath for 60 minutes and further its antioxidant activity was analyzed using Hydroxyl radical scavenging activity. It showed 65% of inhibition when compared to Ascorbic acid which showed only 41%, which confirms that, the Long pepper proteins are more thermal stable (8). Further we investigated the toxicity of proteins of Long pepper using human cells. It was observed that, there was no decrease in the number of viable cells when Long pepper proteins were added. The potential of the extract to quench hydrogen peroxide and nitric oxide radicals in vitro could justify its cytoprotective action in the processes of the cytoprotective effect of the extract, such as the direct scavenging of free radicals (hydroxyl radicals, butoxyl radicals, and nitric oxide) generated by the oxidative stress induced by hydrogen peroxide and tert-butyl hydroperoxide, the increase of intracellular levels of antioxidant enzymes and glutathione, or the reduction of hydrogen to water molecules against hydrogen peroxide and tert-butyl hydroperoxide. These results clearly indicate that proteins of Long pepper are non-toxic for cells. In conclusion, the obtained results of proteins of Long pepper are very promising and interesting and hence further studies about its medicinal properties need to be done.

CONCLUSIONS

P.longum is one of the most popular spices in the world, with a day-to-day use and growing fame as a source of bioactive molecules with pharmacological properties. *P.longum* has been reported to possess undeniable anticancer potential in different cancer cell lines and animal mod. Although piperine is the major active constituent of long pepper and the most characterized in its multiple mechanisms of action counteracting cancer development, other constituents, such as piperlongumine, pellitorine, and kusunokinin, have been demonstrated to have remarkable anticancer properties.

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